KITS AND METHODS FOR MAKING LARGE RECOMBINANT POLYNUCLEOTIDES

PARENT CASE TEXT

This patent application claims priority to U.S. Provisional Patent Application No. 60/533,943, which was filed on January 2, 2004.

SEQUENCE LISTING

A paper copy of the sequence listing and a computer readable form of the same sequence listing are appended below and herein incorporated by reference. The information recorded in computer readable form is identical to the written sequence listing, according to 37 C.F.R. 1.821 (f).

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to in vitro methods of making large recombinant polynucleotides useful in the production of genome scale vectors, gene therapies, vaccines and research tools. A method of manipulating a parent polynucleotide by generating polynucleotide fragments with asymmetric single stranded sticky ends that can reassemble into the correct sequence. This discovery enables the simple production of recombinant polynucleotide molecules in vitro, such as the production of large gene therapy vectors.

Background of the Invention

The explosion in the health care, pharmaceutical, life sciences, and biotechnology industries has been fueled in large part by advances in recombinant polynucleotide technology. A cornerstone to recombinant polynucleotide technology is the ability to manipulate nucleic acid sequences, to construct vectors and to introduce those vectors into cells to produce a desired outcome. Site-directed mutagenesis, which is the deliberate introduction of a change into a naturally occurring polynucleotide sequence, is a major component to this cornerstone technology. Most site-directed mutagenesis protocols utilize the polymerase chain reaction to introduce custom

primers, which contain an altered nucleic acid sequence, into the product nucleic acid see PCR Primer (Dveksler, D. C. a. G. (1995). PCR Primer: A Laboratory Primer, Cold Spring Harbor Laboratory Press) which is incorporated herein by reference.

A major limitation to the optimal implementation of recombinant polynucleotide technology is the difficulty of manipulating small or large pieces of polynucleotides, i.e., <500 nucleotides (nt) or >5,000 nt, respectively. Applications in functional genomics, vaccine development and gene therapy often require the construction of large viral vectors. For example, adenoviral vectors, which are used in the construction of human gene therapy vectors, are approximately 36,000 nt base pairs in length. Such large virus-based vectors are usually constructed according to a three-step protocol. The first step involves the *in vitro* construction of a part or parts of the vector by standard plasmid- or cosmid-based cloning methods. The second step utilizes homologous recombination of those fragments in a host cell to produce plaques which contain the final recombinant viral vectors. The third step is screening for plaques that contain the recombinant vector of interest and subsequently purifying the correct vector construct.

It is important to note that, since viruses can recombine with the host genome, individual plaques do not contain a homogeneous population of vectors, but rather contain a heterogeneous mixture of vectors. Francois et al. (J Virol 75(11): 5288-301, 2001) described a cosmid-based method of producing an avian adenovirus that results in homogenous vectors. That method utilizes a three-step (*supra*) homologous recombination approach to first construct the virus vector, followed by a phage lambda packaging step to amplify the vector. That method thus offers a way to make a homogeneous supply of vectors, once the parent vector has been constructed. Overall, this three-step protocol is inefficient and technically difficult. The following references, which are incorporated herein by reference, describe the current state of the art of virus vector construction: Hardy et al., J. Virol. 71:1842-1849 (1997); Giampaoli et al., J Gene Med. 4(5):490-7 (2002); Sauter S.B. BioProcessing Journal May:56-61 (2003); Rodriguez et al., Ed. (1988) Vectors: A Survey of Molecular Cloning Vectors and their Uses. Butterworth Publishers; Curiel et al., Ed. (2002) Vector Targeting for Therapeutic Gene Delivery. John Wiley & Sons Publishers.

In general, four types of vectors are commonly used in the construction of recombinant biomolecules: plasmids, bacteriophage and cosmids, and artificial

chromosomes. While plasmids, bacteriophage and cosmids have a higher transformation efficiency and ease of purification, the maximum size of the polynucleotide that can be cloned in these vectors is 20, 25 and 45 kb (kilobases) , respectively (Hohn, B. (1979), Methods Enzymol 68: 299-309 and Berg, P. (1981), Science 213(4505): 296-303). For insert sizes of 100 kb-2000 kb, artificial chromosomes are used. Artificial chromosomes have been derived from yeast (yeast artificial chromosomes: YACs), bacteria (bacterial artificial chromosomes: BACs, and P1-derived artificial chromosomes: PACs), and mammals (mammalian artificial chromosomes: MACs), such as humans (human artificial chromosomes: HACs). Those vectors include elements derived from chromosomes that are responsible for replication and maintenance, and are capable of stably maintaining large genomic polynucleotide fragments. However artificial chromosomes are known to be unstable and tend to delete regions of polynucleotides. Furthermore, purification of artificial chromosomes is complex due to their structural similarity to native chromosomes of the host cell genome (Monaco et al. (1994). Trends Biotechnol 12(7): 280-6).

To accomplish site-directed mutagenesis of large gene targeting vectors, as opposed to subcloning to obtain smaller plasmids, DeTogni et al. (De Togni, P. (1997). Biotechniques 22(3): 426-8, 430) developed a protocol for introduction of mutations into large plasmids. In this protocol three oligonucleotide primers are required. The first primer contains the desired mutation for the gene of interest, the second primer contains a mutation in a unique, nonessential restriction site and a third primer is used to intercalate between the first and second primers, reducing the length of the gap in the plasmid that the DNA polymerase needs to fill in to produce the mutagenized plasmid (De Togni 1997). However, mutagenesis efficiency is low using that method due to the large size of the plasmid.

PCR has also been used to generate site-specific mutants (Jones et al. (1991) Methods: A Companion to Methods in Enzymology, vol. 2, no. 1, February, pp. 2-10, U.S. Pat. No. 5,286,632). This method involves use of PCR primers to extend two DNA sequences from two separate circular plasmids. The two extended PCR products can be combined to form a circular plasmid and the mutations introduced by use of nucleotide changes within the primer sequence or by incorporating a sequence from the complementary region of the primers to the plasmid.

SUMMARY OF THE INVENTION

The inventor has made the surprising discovery that polynucleotide molecules, which have been digested using enzymes that generate asymmetric sticky ends to produce multiple polynucleotide fragments in which each individual polynucleotide fragment has two stick-ends that are mutually non-complemetary, can reassemble into the correct sequence. This discovery enables the simple production of recombinant polynucleotide molecules in vitro, such as the production of large gene therapy vectors, without having to go through cumbersome, inaccurate and inefficient in vivo recombination and selection steps.

Upon digestion by enzymes that generate asymmetric sticky ends, the ends of the resulting fragments will be blunt or sticky, depending on the parent polynucleotide sequence used. A circular parent polynucleotide digested by such an enzyme will always generate fragments with two sticky ends that are mutually non-complementary. A linear parent polynucleotide that is cleaved only once by such an enzyme will result in two polynucleotide fragments with one fragment having a blunt end and a sticky end and the other fragment having a sticky end and a blunt end, where the sticky ends are mutually non-complementary. A linear parent polynucleotide that is cleaved multiple times by such an enzyme will result in more than two polynucleotide fragments with one fragment having a blunt end and a sticky end, another fragment having a sticky end and a blunt end and all remaining fragments having two sticky ends, where all sticky ends are mutually non-complementary.

In one embodiment, the present invention is directed to a method for generating a recombinant polynucleotide. The method comprises the steps of (a) generating a mixture of linear polynucleotide fragments derived from a parent polynucleotide, wherein each polynucleotide fragment (i) is double-stranded across at least 50% of its length, (ii) has a first single-stranded sticky end at the first end and a second single-stranded sticky end at the second end, such that (iii) in an average of at most 1 in 256 of linear polynucleotide fragments generated, the first single-stranded sticky end is not complementary to the second single-stranded sticky end; (b) introducing to the mixture at least one additional polynucleotide, which (i) is linear, (ii) is double-stranded across at least 50% of its length, (iii) has a first single-stranded sticky end at the first end and a second single-stranded sticky end at the second end, such that (iv)

in an average of at most 1 in 256 of linear polynucleotide fragments generated, the first single-stranded sticky end is not complementary to the second single-stranded sticky end to the fragments, and (v) the first and second single-stranded sticky ends of the additional polynucleotide are compatible to one or more single-stranded sticky ends of one or more linear polynucleotide fragments of the mixture; and (c) ligating the fragments and the additional polynucleotide together to produce the recombinant polynucleotide.

A preferred parent polynucleotide is a large polynucleotide vector, such as a viral genome or artificial chromosome. More preferably, the parent polynucleotide is a gene therapy vector, such as an adenovirus vector. Parent polynucleotide is used in the broadest sense and includes not only linear types of sequences, but three-dimensional objects (such as nanoboxes) constructed of nucleic acids. The instant method may be used in the assembly of these types of nano-structures.

A preferred additional polynucleotide encodes a protein that is not normally encoded by the parent polynucleotide or comprises a mutation that interferes with the production of a protein that is otherwise naturally encoded by the parent polynucleotide. The additional polynucleotide may be synthetic, such as an artificial chromosome or synthetic oligonucleotide, or naturally occurring. The additional polynucleotide may be obtained from any source, such as for example by artificial synthesis, bacteria, fungi, protists, algae, plants and animals. Alternatively, the additional polynucleotide may be not only only linear types of sequences, but three-dimensional objects (such as nanoboxes) constructed of nucleic acids (*supra*).

In a preferred embodiment, the polynucleotide fragments are generated by digesting the parent polynucleotide with one or more asymmetric endonucleases (see Table 1 for non-limiting examples of useful asymmetric endonucleases).

In another embodiment, the invention is directed to kits that are useful in the production of recombinant polynucleotides. Such kits may bed used for (1) gene therapy vectors (therapeutic kits), (2) research tools (bench kits for making vectors), (3) vaccine development (kit for vaccine development) or (4) any other applications that utilize recombinant polynucleotides. In a preferred embodiment, the kits would comprise at least one polynucleotide fragment having a unique 3' and 5' asymmetric single stranded sticky ends, an enzyme, such as a ligase, buffers, and small single-

stranded polynucleotides, such as primers or linkers. More preferably, the kits would comprise multiple polynucleotide fragments that span a portion or all of a useful parent polynucleotide. Most preferably, the parent polynucleotide is an adenovirus genome.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 shows insertion of a polynucleotide into a recognition site for the asymmetric endonuclease BstXI.

Fig. 2 depicts how a DNA sequence cleaved with an asymmetric endonuclease will self-assemble in the correct order and with the correct orientation upon introduction of a DNA ligase.

Fig. 3 is the map of pFG140 with BstXI and HindIII restriction sites.

Fig 4 is a linear schema of the restriction map of pFG140 showing BstXI and HindIII sites with the predicted DNA bp size.

Fig 5, Panel A is a Southern blot examining digestion of plasmids pFG140 (wild type) and pFG5 (mutant) with BstXI or HindIII. The fragments were run on a 1% agarose gel in lanes 1 and 2 or lanes 3 and 4, respectively. Lamda HindIII makers were run in lane 5. Position of pFG140 and pFG5 BstXI and HindIII fragments are shown to the left and right of Panel A, respectively. The asterisks indicate fragments that contain DNA from pUC118 (accession number U07649) that encodes the lac I promoter, the poly cloning site and the lacZ mutation (nucleotides 3158-515) and seven additional nucleotides from the primer set used to amplify this DNA fragment from pUC118. The sizes of Lambda HindIII fragments are 23071, 9416, 6682, 4359, 2322, 2027, 564. Panel B is a Southern blot showing an overexposured image of the same gel as Fig 5, Panel A to visualize the pFG140-322 bp and pFG5-519 bp BstXI fragments, which are indicated by arrows.

Fig. 6 depicts the bracketed and sequential method of assembling a recombinant polynucleotide.

Figure 7 depicts an ethidium stained gel of the step wise assembly of BstX1 digested pFG140, according to the scenario depicted in figure 6.

Figure 8 depicts the ligation mixture of fragments 1 and 2.

Figure 9 depicts the ligation mixture of fragments 3 and 4.

Figure 10 depicts the ligation mixture of fragments 5 and 6.

Figure 11 depicts the ligation mixture of fragments 7 and 8.

DETAILED DESCRIPTION OF THE INVENTION

According to the current state of the art, the probability of constructing a correct recombinant DNA vector approaches zero, $P(x) \approx 0$. It is successful due to the powerful drug/viral selection systems that are cloned into these DNAs. In other words, traditional recombinant vector construction relys on powerful tools to pull "a needle from a haystack."

The inventor discloses herein a revolutionary method of constructing a recombinant vector, wherein the probability of constructing the correct recombinant vector, by in vitro ligation methods, approaches unity. The instant method utilizes the interesting property of restriction endonucleases that recognize "interrupted palindromes." These particular restriction enzymes recognize a particular sequence that is some distance from the site of cleavage, wherein the site of cleavage may have any sequence (e.g., random). This property allows for the creation of "sticky ends" that can only bind in a particular orientation with a particular binding mate, thereby facilitating the assembly of polynucleotide fragments in a particular order and orientation.

To illustrate this concept, a double stranded circular DNA having a two BamH1 sites (Bam HI cuts within the recognition site palindrome to generate identical sticky ends at any and all sites), upon digestion with BamH1, will generate two fragments. Given the palindromic nature of the BamH1 sticky end, the two fragments can reassemble in any one of two orientations, which gives a 50% chance of getting the correct sequence back. Conversely, a double stranded circular DNA having a two BstX1 sites (SEQ ID NO:1: CCANNNNN^NTGG, wherein the carrot ^ represents the cut position and N can be any nucleotide), upon digestion with BstX1, will generate two fragments. Given the variable nature of the BstX1 sticky end, each of the two fragments will have a different sticky end overhang sequence at each end, which permits the reassembly of the two fragments in only one orientation, which gives a 100% chance of getting the correct sequence back. See figure 1.

Thus, the current invention is based upon the discovery of an effective and efficient process for cloning and selection of polynucleotide sequences based on the

use of restriction enzymes that recognize interrupted palindromes. After cleavage of a parent polynucleotide sequence with an asymmetric endonuclease, an additional polynucleotide with sticky ends, which are complementary to a subset of sticky ends in the pool of polynucleotide fragments derived from the parent polynucleotide, is inserted into the parent polynucleotide sequence to create a recombinant polynucleotide. An important aspect of the invention is that with asymmetric single stranded sticky ends, with very few exceptions, a polynucleotide fragment can only enter in one orientation. Furthermore, multiple insertions of multiple additional polynucleotides can be obtained by this method. While the use of restriction enzymes that recognize interrupted palindromes (a.k.a. asymmetric endonucleases) in cloning is known in the art, it is also known in the art to which this invention is directed that a recombinant product cannot be recut with the same asymmetric endonuclease since the interrupted palindromic site is destroyed (Siegal et al. (1996), BioTechniques 21: 619-622). In the claimed method, the interrupted palindromic site is not destroyed and the recombinant product may be re-cut.

In one embodiment, the invention is directed to a method of assembling a recombinant polynucleotide comprising the step of mixing together polynucleotide fragments, each polynucleotide fragment having a different overhanging sequence ("unique sticky end") at each end (or alternatively a blunt end at one end and a unique sticky end ant the other end), allowing those polynucleotide fragments to assemble, and ligating the fragments to produces a recombinant polynucleotide. See figure 2. In a preferred aspect of this method, especially in the case of assembling a long or complex recombinant polynucleotide, subsets of fragments are allowed to self assemble and ligate to form intermediate recombinant fragments, then the intermediate recombinant fragments are combined, allowed to self-assemble and ligate to form a yet more complex and longer recombinant polynucleotide. This method is exemplified in figure 6, which depicts the scenario in which two adjacent fragments are sequentially assemble over three steps to produce the final correct recombinant polynucleotide. (Figure 6).

This method is especially but not solely applicable to manipulating long polynucleotide sequences, such as viral sequences (e.g., adenovirus, Hepesvirus, Pox virus), bacterial sequences, plant sequences, and other large cloned polynucleotide sequences (e.g., YACs, BACs, PACs and MACs).

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods or materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

"Polypeptide", as used herein, means any artificial or naturally occurring molecule comprising two or more amino acids joined together by an amide bond, including, for example, simple dipeptides, single subunit polypeptides, and complex post-translationally modified proteins.

"Asymmetric endonuclease" is defined as a restriction enzyme, which cuts DNA at a specific site defined by a local nucleotide sequence (generally that local nucleotide sequence is an interrupted palindrome) to generate asymmetric single stranded sticky ends. Non-limiting examples are shown in Table 1.

"Asymmetric single stranded sticky ends", as used herein, refer to a set of two single-stranded polynucleotide sequences located at the ends of a linear polynucleotide that is generally double-stranded, wherein the two single-stranded polynucleotide sequences are mutually non-complementary. Asymmetric single stranded sticky ends are generally generated when an asymmetric endonuclease cuts a polynucleotide at interrupted palindrome. See also Table 1.

"Interrupted palindrome" or "interrupted palindromic site" is defined as a nucleotide sequence with unspecified nucleotides between the flanking nucleotides that comprise the palindrome. Endonucleases, which recognize interrupted palindromes (i.e., asymmetric endonucleases), generally cut in the region of unspecified nucleotides. Non-limiting examples are shown in Table 1.

"Polynucleotide fragment" generally refers to a portion of a larger parent polynucleotide released by cleaving (a.k.a. cutting) the larger parent polynucleotide. Alternatively, a polynucleotide fragment may be synthesized based upon a portion of a nucleotide sequence of a parent or larger polynucleotide.

"DNA ligase" is defined as an enzyme which can catalyze the formation of a phosphodiester covalent bond between a 3'-OH and a 5'-phosphate of two adjacent polynucleotides.

TABLE 1: Example asymmetric endonucleases their cleavage sites. (N can be G, C, T or A. ^ shows where the DNA cleavage occurs).

Restriction Enzyme	Sequence	Sequence Identifier
BstXI	CCANNNNN^NTGG	SEQ ID NO:1
BbvI	GCAGCNNNNNNNN^	SEQ ID NO:2
BglI	GCCNNNN^NGGC	SEQ ID NO:3
BpmI	CTGGAGNNNNNNNNNNNNNNNN^	SEQ ID NO:4
Bpu10I	CC^TNAGC	SEQ ID NO:4
BpuEI	CTTGAGNNNNNNNNNNNNNNNN^	SEQ ID NO:6
BsaI	GGTCTCN^	SEQ ID NO:7
BsaBI	GATNN^NNATC	SEQ ID NO:8
BsaJI	C^CNNGG	SEQ ID NO:9
BseRI	GAGGAGNNNNNNNNNN^	SEQ ID NO:10
BsgI	GTGCAGNNNNNNNNNNNNNNN^	SEQ ID NO:11
BsiI	CCNNNNN^NNGG	SEQ ID NO:12
BsmI	GAATGCN^	SEQ ID NO:13
BsmAI	GTCTCN^	SEQ ID NO:14
BsmBI	CGTCTCN^	SEQ ID NO:15
BsmFI	GGGACNNNNNNNNNN^	SEQ ID NO:16
BspMI	ACCTGCNNNN^	SEQ ID NO:17
BsrI	ACTGGN^	SEQ ID NO:18
BsrDI	GCAATGNN^	SEQ ID NO:19
BssKI	^CCNGG	SEQ ID NO:20
BstAPI	GCANNNN^NTGC	SEQ ID NO:21
BstEII	G^GTNACC	SEQ ID NO:22
BstF5I	GGATGNN^	SEQ ID NO:23
Bsu36I	CC^TNAGG	SEQ ID NO:24
Cac8I	GCN^NGC	SEQ ID NO:25
DdeI	C^TNAG	SEQ ID NO:26
DraII	RG^GNCCY	SEQ ID NO:27
DraIII	CACNNN^GTG	SEQ ID NO:28
DrdI	GACNNNN^NNGTC	SEQ ID NO:29
EarI	CTCTTCN^	SEQ ID NO:30
EciI	GGCGGANNNNNNNNNN^	SEQ ID NO:31
EcoO109I	RG^GNCCY	SEQ ID NO:32
FauI	CCCGCNNNN^	SEQ ID NO:33
FokI	GGATGNNNNNNNNN^	SEQ ID NO:34
HgaI	GACGCNNNNN^	SEQ ID NO:35
HhaII	G^ANTC	SEQ ID NO:36
HinfI	G^ANTC	SEQ ID NO:37
HphI	GGTGANNNNNNNN^	SEQ ID NO:38
Hpy188I	TCN^GA	SEQ ID NO:39

Hpy188III	TC^NNGA	SEQ ID NO:40
MboII	GAAGANNNNNNNN^	SEQ ID NO:41
MnlI	CCTCNNNNNNN^	SEQ ID NO:42
MslI	CAYNN^NNRTG	SEQ ID NO:43
NlaIV	GGN^NCC	SEQ ID NO:44
PflFI	GACN^NNGTC	SEQ ID NO:45
PflMI	CCANNNN^NTGG	SEQ ID NO:46
PleI	GAGTCNNNN^	SEQ ID NO:47
PshAI	GACNN^NNGTC	SEQ ID NO:48
SapI	GCTCTTCN^	SEQ ID NO:49
Sau96I	G^GNCC	SEQ ID NO:50
ScrFI	CC^NGG	SEQ ID NO:51
SfaNI	GCATCNNNNN^	SEQ ID NO:52
TspRI	NNCASTGNN^	SEQ ID NO:53
Tth111I	GACN^NNGTC	SEQ ID NO:54
XcmI	CCANNNNN^NNNNTGG	SEQ ID NO:55
XmnI	GAANN^NNTTC	SEQ ID NO:56

In one embodiment, the invention is drawn to a way to manipulate polynucleotide molecules by generating polynucleotide fragments with asymmetric single stranded sticky ends that can reassemble into a useful sequence.

Asymmetric sticky ends may be generated by, but not limited to, use of an asymmetric endonuclease (Sambrook et al., (1989) Molecular cloning, A Labroratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press), see Table 1 for examples. The asymmetric endonuclease sites (a.k.a. interrupted palindrome) of a polynucleotide may be identified by conventional restriction enzyme mapping techniques (Sambrook, 1987), by analysis of sequence information for that gene, or by introduction of desired restriction sites into a nucleic acid sequence by site-directed mutagenesis or by incorporation of silent or other mutations.

An advantage of using polynucleotide fragments with asymmetric single stranded sticky ends to manipulate a polynucleotide or to create a recombinant polynucleotide is exemplified in figure 1, which depicts how the introduction of an excess of polynucleotide with asymmetric single stranded sticky ends is inserted into a correct orientation and at the correct location within the "backbone" (parent) polynucleotide. Further, Figure 2 depicts how a polynucleotide cleaved with an asymmetric endonuclease will self-assemble in the correct order and with correct orientation, and upon the introduction of a ligase to reform the polynucleotide having the original sequence. Additionally, the skilled artisan would readily appreciate that multiple polynucleotide fragments, each fragment having mutually non-complementary sticky

ends, may be introduced sequentially and associate into the correct orientation and at the correct location.

The current construction of adenoviruses is a cumbersome and lengthy process. The method disclosed herein may be used to quickly and easily generate a recombinant adenovirus. This recombinant adenovirus may be used as a vector for gene therapy (Zabner et al. (1993), Cell 75:207-216 and Crystal et al. (1994), Nature Genetics 8:42-51), for possible live vaccines (Chanda et al. (1990), Virology 175:535-547) and as a research tool to study gene function in cells (Ragot et al. (1993), Nature 361:647-650).

Furthermore, the instant method may be used to generate other recombinant viruses such as a recombinant poxvirus, herpes virus, polyomaviruses, hepadnaviruses, orthomyxovirus, rhabdoviruses and alphaviruses. Like adenovirus, these recombinant viruses may then also be used for gene vehicle delivery, vaccines, development of antibodies and research. The instant method may also be used to generate a plant vector useful in the production of beneficial products. Such products may include, but are not limited to, the production of vitamins or other beneficial nutrients, pesticide resistance compounds, herbicide resistance compounds, stress (as in heat or drought) protective compounds, and nutrients beneficial to livestock growth and development and human health. Additionally, the recombinant polynucleotides generated via the instant invention may be used in the cell-based production of therapeutic compounds and research tools.

EXAMPLE 1

The inventor has succeeded in inventing a method for cloning and selection of polynucleotide sequences based on the generation and use of polynucleotide fragments with asymmetric single stranded sticky ends. In this example, digestion of the plasmid pFG140 with BstXI resulted in multiple polynucleotide fragments with asymmetric single stranded sticky ends. Upon addition of an additional polynucleotide with asymmetric single stranded sticky ends that are complementary to the sticky ends generated by BstXI, namely DNA from pUC118 (accession number U07649), the fragments were sealed by DNA ligase to form the plasmid pFG5. The polynucleotide fragments generated by digestion with BstXI were visualized by Southern Blot (Fig 5, Panel A and B). To confirm that the new polynucleotide

oriented it self correctly the plasmid was then cleaved by HindIII to generate polynucleotide fragments of the expected size. The fragments visualized by Southern Blot further showed that the polynucleotide did indeed insert correctly. Figure 3 depicts a circular restriction map of pFG140 with BstXI and HindIII restriction sites while the linear schema of Figure 4 shows the predicted polynucleotide size. These polynucleotide sizes correspond in size to the fragments generated by BstXI or HindIII cleavage, as shown by the aforementioned Southern Blot (Fig 5, Panel A and B).

As various changes could be made in the above methods, compositions and working examples without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

EXAMPLE 2

The multistep, bracketed approach to assembling a recombinant polynucleotide in the correct sequence (fig. 6) was demonstrated using BstX1 digested pFG140 (figure 7). The circular map of BstX1 is presented in figure 3 and the BstX1 fragments are depicted in the top row of figure 4. After digesting with BstX1, eight polynucleotide fragments were generated having sizes of 4067 (fragment 1), 5883 (fragment 2), 5251 (fragment 3), 4254 (fragment 4), 11921 (fragment 5), 1399 (fragment 6) and 4924 (fragment 7) nucleotide base pairs (nt. or bp.). Each of the fragments were gel purified (as is readily known in the molecular biological arts). Fragments 1 and 2, 3 and 4, 5 and 6, and 7 and 8 were respectively combined, and ligated. The ligation products were run out on a gel (figure 7).

The ligation product of 1 and 2 is depicted both graphically and by gel analysis in figure 8. Both fragments 1 and 2 encode non-palindromic sticky-ends. Only two of these non-palindromic sticky-ends are complementary, so in theory, only one ligation product should be observed. In fact, the only ligation product, a 4586 bp product, was observed.

The ligation product of 3 and 4 is depicted both graphically and by gel analysis in figure 9. Both fragments 3 and 4 encode non-palindromic sticky-ends. Only two of these non-palindromic sticky-ends are complementary, so in theory, only one ligation

product should be observed. In fact, the only ligation product, a 11,134 bp product, was observed.

The ligation product of fragments 5 and 6 is depicted both graphically and by gel analysis in figure 10. Both fragments 5 and 6 each encode one non-palindromic sticky-end and one palindromic sticky-end that is complementary to the other fragment's palindromic sticky-end. In theory, ligation of these fragments should produce three ligation products: a fragment 6-6 dimer, a fragment 5-6 dimer, and a fragment 5-5 dimer. These three ligation products were observed migrating at 23,842 bp, 16845 bp, and 9848 bp. See figure 10.

The ligation product of 7 and 8 is depicted both graphically and by gel analysis in figure 11. Both fragments 7 and 9 encode non-palindromic sticky-ends. Only two of these non-palindromic sticky-ends are complementary, so in theory, only one ligation product should be observed. In fact, the only ligation product, a 6,323 bp product, was observed.